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Bauhinia forficata: AVALIAÇÃO DOS EFEITOS METABÓLICOS E ANTIOXIDANTES EM RATOS DIABÉTICOS

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Dissertação apresentada ao programa de Pós-graduação em farmacologia da Universidade Federal do Paraná como Requisito parcial para obtenção do título de Mestre em Farmacologia.

Orientadora: Prof[a].Dra. Alexandra Acco

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PARECER

A Comissão Examinadora da Dissertação de Mestrado intitulada "*Bauhinia forficata*: AVALIAÇÃO DOS EFEITOS METABÓLICOS E ANTIOXIDANTES EM RATOS DIABÉTICOS", de autoria da pós-graduanda **LIANA DE OLIVEIRA GOMES,** sob orientação da Prof.ª Dr.ª Alexandra Acco e composta pelos professores: Prof.ª Dr.ª Alexandra Acco (Presidente – Farmacologia – UFPR); Prof. Dr. Michel Fleith Otuki (Farmacologia – UFPR) e Prof.ª Dr.ª Sílvia Maria Suter Correia Cadena (Bioquímica – UFPR), reuniu-se e, de acordo com o Regimento Interno do Programa de Pós-Graduação em Farmacologia, a pós-graduanda foi Aprovada. Para a devida publicação o trabalho deverá sofrer as modificações sugeridas, que serão conferidas por sua orientadora. Em Curitiba, 19 de setembro de 2013.

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Esta dissertação é apresentada em formato alternativo – artigo para publicação – de acordo com as normas do Programa de Pós-Graduação em Farmacologia da Universidade Federal do Paraná, constando de uma revisão de literatura, objetivos do trabalho e um artigo científico abordando os experimentos realizados, com resultados e discussão, além da conclusão. O artigo foi formatado conforme as normas propostas por periódicos de circulação internacional.

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“Os grandes feitos são conseguidos não pela força, mas pela perseverança”.

Samuel Johnson

RESUMO

Introdução e Objetivo: O diabetes é um grupo de doenças metabólicas caracterizado por hiperglicemia associada a complicações, disfunções e falência de vários órgãos. A planta *Bauhinia forficata* (BF), conhecida como "pata-de-vaca", é popularmente usada em diabetes para reduzir a glicemia. Este trabalho objetivou avaliar os efeitos metabólicos, hipoglicemiantes e antioxidantes do extrato etanólico de folhas de BF em ratos normoglicêmicos e com diabetes induzida por estreptozotocina.

Material e Métodos: Fígados de ratos normoglicêmicos e diabéticos foram perfundidos com extrato etanólico de BF (40 mg L^{-1}) e o metabolismo hepático da glicose foi avaliado em estado alimentado e em jejum (L-glutamina como substrato). Em adicional, ratos foram divididos em 4 grupos e tratados diariamente: normoglicêmicos tratados com veículo, normoglicêmicos tratados com BF (300 mg kg^{-1}, oral), diabéticos tratados com veículo e diabéticos tratados com BF. A glicemia foi medida agudamente e após 7 dias de tratamento; e parâmetros de bioquímica plasmática, estresse oxidativo hepático, glicogênio hepático e histologia pancreática foram avaliados após este período. A atividade antioxidante de extratos de BF foi testada *in vitro*.

Resultados: O metabolismo hepático diferiu entre ratos diabéticos e normoglicêmicos. A infusão de BF em ratos diabéticos alimentados e em jejum reduziu a produção de glicose e de lactato, respectivamente. A BF também induziu ao aumento transitório da produção de piruvato em ambos, ratos diabéticos e normoglicêmicos. Entretanto, o tratamento com BF não afetou a glicemia, os níveis de glicogênio hepático e o número de ilhotas de Langerhans no pâncreas. Ratos diabéticos que receberam o extrato apresentaram elevação das transaminases plasmáticas (ALT e AST). O extrato de BF exibiu intensa atividade antioxidante *in vitro*, enquanto o efeito *in vivo* foi mínimo.

Conclusões: Nas presentes condições experimentais o extrato etanólico de BF interferiu na glicólise, na gliconeogênese e na produção de lactato a partir da L-glutamina. No entanto, o tratamento não suprimiu a hiperglicemia de ratos diabéticos. Apesar do intenso efeito antioxidante *in vitro*, a atividade antioxidante hepática foi minimamente modificada pelo tratamento com BF.

Estudos complementares são necessários para esclarecer os efeitos metabólicos e toxicológicos da BF, incluindo análises com outras fontes gliconeogênicas além da L-glutamina. Este é o primeiro estudo que demonstrou os efeitos da BF no metabolismo hepático.

Palavras-chave: *Bauhinia forficata*, diabetes, perfusão hepática, metabolismo hepático, glicose, ratos.

ABSTRACT

Introduction and Aim: Diabetes is a group of metabolic diseases characterized by hyperglycemia associated with complications, dysfunction and failure of various organs. *Bauhinia forficata* (BF), known as “cow’s foot”, is popularly used in diabetes to reduce the glycemia. This work aimed to evaluate the metabolic, hypoglycemic and antioxidant effects of the ethanolic extract of BF leaves in streptozotocin-induced diabetic rats and normoglycemic rats.

Material and Methods: The livers of normoglycemic and diabetic Wistar rats were perfused with BF extract (40 mg L^{-1}) and hepatic glucose metabolism under fed or fasting conditions (L-glutamine as the substrate) was evaluated. Experimental rats were divided into 4 groups and treated daily: vehicle-treated non-diabetic rats, BF-treated (300 mg kg^{-1}, oral) non-diabetic rats, vehicle-treated diabetic rats, and BF-treated diabetic rats. Glycemia was measured acutely and after 7 days of treatment; plasma biochemistry, hepatic oxidative stress, hepatic glycogen, and hepatic and pancreatic histology were evaluated after this period. The antioxidant activity of the BF extracts was tested *in vitro*.

Results: Hepatic metabolism differed between diabetic and normoglycemic rats. Liver infusion of BF in fed and fasted diabetic rats reduced glucose and lactate production, respectively. BF also induced a transitory increase in pyruvate production in both fasted diabetic and normoglycemic rat livers. However, BF treatment did not affect glycemia, hepatic glycogen levels and the number of pancreatic Langerhans islets. Plasma transaminases (ALT and AST) increased in diabetic animals receiving the extract. The BF ethanolic extract exhibited antioxidant activity *in vitro*, while the *in vivo* effect was minimal.

Conclusions: In these experimental conditions the ethanolic extract of BF interferes in the hepatic glucolysis, gluconeogenesis, and in the lactate production from the L-glutamine. However, the treatment did not suppress the hyperglycemia in diabetic rats. Despite the intense antioxidant effect *in vitro*, the hepatic antioxidant activity was minimally modified by the BF treatment. Further studies are necessary to better assess its metabolic and toxicological effects, including analysis of other sources of gluconeogenesis than L-glutamine. This is the first study to demonstrate the effects of BF on hepatic metabolism.

Key words: *Bauhinia forficata*, streptozotocin-induced diabetes, liver perfusion, hepatic metabolism, glucose, rats.

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LISTA DE ABREVIATURAS

DM – Diabetes Mellitus

RL – Radicais livres

SOD – Superóxido dismutase

GPx – Glutationa peroxidase

Cat – Catalase

LDL- Lipoproteína de baixa densidade

AGEs – produtos finais da glicação avançada

DPP-4 - Dipeptidilpeptidase IV

ATP – Adenosina trifosfato

GLUTs – Transportadores de glicose

NAD - Nicotinamida adenina dinucleotídeo

FAD – Dinucleotídeo de flavina e adenina

NADH - Nicotinamida adenina dinucleotídeo reduzida

FADH- Dinucleotídeo de flavina e adenina reduzida

LDH – Lactato desidrogenase

DAG – Diacilglicerol

PKC – Proteína quinase C

BF – *Bauhinia forficata*

HDL- Lipoproteína de alta densidade

DPPH - 1,1-difenil-2-picrilhidrazil

ARTIGO CIENTÍFICO

D1TM – Diabetes type 1

D2TM – Diabetes type 2

BF – *Bauhinia forficata*

Hex – Hexane

DCM – Dichloromethane

AcOEt - ethyl acetate

EtOH – ethanol

$NMRH^{1}$ - Nuclear magnetic resonance

STZ – Streptozotocin

KH - Krebs/Henseleit-bicarbonate

Cat – Catalase

GST - Glutathione-S-transferase

SOD - Superoxide dismutase

LPO - Lipid peroxidation

GSH - Reduced glutathione

ALT - Alanine aminotransferase

AST - Aspartate aminotransferase

HE - Hematoxylin and eosin (HE)

DPPH - Radical 2,2-diphenyl-1-picrylhydrazyl

NADH – Nicotinamide adenine dinucleotíde reduced

NAD - Nicotinamide adenine dinucleotíde

DKA - Diabetic ketoacidosis

HepG2 – Hepatocellular carcinoma cells

SUMÁRIO

1. INTRODUÇÃO

1.1 O diabetes e suas implicações

O diabetes é uma doença crônica, caracterizada por hiperglicemia, em que ocorre deficiência absoluta ou relativa na secreção ou ação da insulina. A insulina é responsável pela regulação da glicemia plasmática, e o aumento da glicemia, efeito comum do diabetes descontrolado, ao longo do tempo leva a sérios danos a vários sistemas orgânicos (Liu, 2013; WHO, 2013).

O Diabetes mellitus (DM) configura-se hoje como uma epidemia mundial, traduzindo-se em um grande desafio para os sistemas de saúde de todo o mundo. O envelhecimento da população, a urbanização crescente e a adoção de estilos de vida pouco saudáveis, como sedentarismo, dieta inadequada e obesidade, são os grandes responsáveis pelo aumento da incidência e prevalência do diabetes em todo o mundo (MOH, 2006). Atualmente, estima-se que 347 milhões de pessoas no mundo tenham diabetes (WHO, 2013). Segundo a Federação Internacional de Diabetes (2012) o número de portadores da doença é crescente, e o Brasil ocupa a 4^{a} posição entre os países com maior prevalência da doença, abrigando 13,4 milhões de pessoas portadoras. Isto corresponde a aproximadamente 6,5% da população entre 20 e 79 anos de idade. Frente a tais números, as consequências humanas, sociais e econômicas do diabetes são devastadoras.

Atualmente, são três os critérios aceitos para o diagnóstico de DM: sintomas de poliúria, polidipsia e perda ponderal acrescidos de glicemia casual acima de 200 mg/dL ou glicemia de jejum igual ou superior a 126 mg/dL. Em caso de pequenas elevações da glicemia, deve-se confirmar o diagnóstico pela repetição do teste em outro dia ou glicemia acima de 200 mg/dL duas horas pós-sobrecarga de 75 g de glicose (GSBD, 2009).

As formas mais frequentes são o diabetes tipo 1 e o diabetes tipo 2. No diabetes tipo 1 ocorre destruição das células beta do pâncreas, comumente através de um processo autoimune ou de forma idiopática, sendo esta menos

comum. Como consequência da perda dessas células observa-se a deficiência absoluta da secreção de insulina. A maior incidência do diabetes tipo 1 ocorre dos 10 aos 14 anos de idade, no entanto indivíduos de qualquer idade podem desenvolver este tipo de diabetes (Gross, 2002). Já o diabetes tipo 2 é mais comum do que o tipo 1, sendo o responsável por cerca de 90% dos casos. É caracterizado por distúrbios na ação e secreção da insulina. A etiologia específica deste tipo de diabetes ainda não está claramente estabelecida. A idade de início do diabetes tipo 2 é variável , sendo mais frequente após os 40 anos de idade (Gross, 2002). Ambas as formas de DM geram um grande impacto econômico nos serviços de saúde, como consequência dos crescentes custos do tratamento de complicações agudas e crônicas relacionadas à doença. Como complicações agudas destacam-se a hipoglicemia, cetoacidose diabética e o coma hiperosmolar. Como exemplos de complicações crônicas podem ser observadas alterações na microcirculação, originando retinopatia e nefropatia; na macrocirculação, levando à cardiopatia isquêmica, doença cerebrovascular e doença vascular periférica, além de neuropatias. As complicações degenerativas mais frequentes são: infarto agudo do miocárdio, arteriopatia periférica, acidente vascular cerebral, microangiopatia, nefropatia e neuropatia (Pasqualotto et al, 2012).

Atualmente, é amplamente aceito que o estresse oxidativo participa do desenvolvimento e progressão do diabetes e de suas complicações (Maritim, 2003). Vários estudos têm demonstrado que o diabetes está associado a um aumento da formação de radicais livres (RL) e à diminuição no potencial antioxidante celular. Isto aconteceria devido à glicosilação de proteínas, auto-oxidação da glicose, deficiência no metabolismo da glutationa e alterações em enzimas antioxidantes, que incluem superóxido dismutase (SOD), glutationa peroxidase (GPx) e catalase (Cat). Estas enzimas contribuem para a eliminação de superóxido, peróxido de hidrogênio, radicais hidroxila e peróxidos lipídicos (Moretoni, 2008; Samarghadian et al, 2013). Níveis excessivamente elevados de radicais livres causam danos a proteínas celulares, lipídeos de membrana e ácidos nucleicos, levando, eventualmente, à morte celular. Dentre os diversos mecanismos apontados como responsáveis por contribuírem para a formação de RL no diabetes, a oxidação da glicose

seria o principal, pois é oxidada em uma reação dependente de metais de transição, sendo convertida a cetoaldeídos reativos ou radicais superóxido. Estes radicais superóxido sofrem dismutação, formando peróxido de hidrogênio, que se não for degradado pela enzima catalase ou pela glutationa peroxidase, na presença de metais de transição, podem levar à produção de radicais hidroxilas extremamente reativos. Os radicais superóxido também podem reagir com o óxido nítrico para formar peroxinitrito. A hiperglicemia também pode promover a peroxidação lipídica das lipoproteínas de baixa densidade (LDL) por uma via dependente de superóxido resultando na geração de radicais livres. Outra fonte de radicais livres no diabetes seria devido à interação da glicose com proteínas, que conduziria à formação de produtos finais da glicação avançada (AGEs). Estes são capazes de inativar enzimas, alterar suas estruturas e funções, bloquear os efeitos antiproliferativos do óxido nítrico, que se acredita ser um mediador de danos nas células betas das ilhotas pancreáticas (Maritim, 2003).

O tratamento do diabetes visa em curto prazo eliminar os sintomas causados pela hiperglicemia, como a poliúria, polidipsia e perda de peso e em longo prazo prevenir as complicações crônicas e eventuais mortes associados ao diabetes descontrolado.

O tratamento do DM1 consiste basicamente na administração de insulina exógena por injeções diárias e também da adesão a várias tarefas de autocuidado (Wu et al, 2013). Em pacientes portadores de DM 2 que não estão adequadamente controlados com dieta e/ou agentes hipoglicemiantes orais, pode ser necessária administração de insulina (MOH,2006)

Os agentes hipoglicemiantes disponíveis atuam por diferentes mecanismos de ação, podem aumentar a secreção de insulina das células β do pâncreas como as sulfoniluréias (glibenclamida), meglitinas (repaglinida) e os derivados da D-fenilalanina (nateglinida). Podem aumentar a ação da insulina como as biguanidas (metformina) que atuam principalmente diminuindo a produção hepática de glicose e as tiazolidinedionas (pioglitazona) que agem aumentando a sensibilidade do músculo, tecido adiposo e fígado à insulina. Há também os fármacos que atuam através da inibição da absorção de carboidratos, os

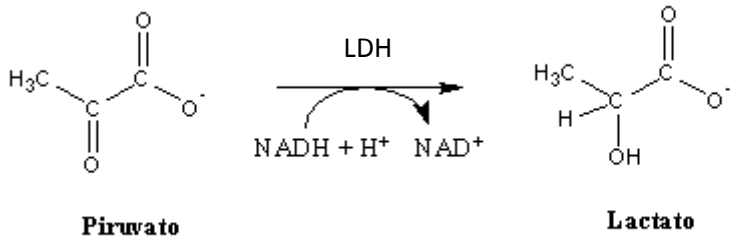
inibidores da α-glicosidase (acarbose) desta forma reduzem os níveis de glicemia pós-prandial predominantemente. Mais recentemente foram inseridos no tratamento do diabetes os fármacos incretinomiméticos ou agonistas GLP, *Glucagon-like peptide*-1 (exenatida). GLP-1 é um peptídeo produzido naturalmente no intestino delgado, que potencializa a estimulação da secreção de insulina. GLP-1 e peptídeos insulinotrópicos dependentes de glicose (GIP), essas incretinas são liberadas com a ingestão de alimentos e sua ação resulta na maior secreção de insulina. Eles são degradados rapidamente pela enzima dipeptidil peptidase (DPP-4) e os hipoglicemiantes chamados de inibidores da DPP-4 (sitagliptina) prolongam a vida destes hormônios e assim também o seu efeito (Nathan et al, 2009).

1.2 Regulação do metabolismo hepático da glicose e o diabetes

A glicose é a principal fonte de energia para todos os tipos celulares de mamíferos, nos quais é responsável pelo provimento de ATP, tanto em condições aeróbicas quanto em condições anaeróbicas. Ela é uma molécula polar, insolúvel na membrana plasmática, e o seu transporte é realizado através de difusão facilitada, portanto a favor de seu gradiente de concentração, e dependente da presença de proteínas transportadoras (GLUTs) na superfície das células (Machado, 1998). Três vias metabólicas estão envolvidas no metabolismo da glicose; primeiro a glicose é oxidada através da via glicolítica, então o produto final da glicólise, representado por piruvato que é lançado no ciclo de Krebs, onde é completamente oxidado para formar seis moléculas de dióxido de carbono. No processo, os carreadores de elétrons NAD^+ e FAD, compostos derivados das vitaminas niacina e riboflavina, respectivamente, são reduzidos a NADH e $FADH_2$ e levam o elétron para a cadeia respiratória, onde a energia é armazenada nas moléculas de ATP e o hidrogênio é utilizado para reduzir oxigênio à água (Salway, 2009). A reação parcial da oxidação da glicose nas células vivas pode ser representada da seguinte forma:

$$\text{Glicose} + 2\ NAD^+\ 2\ ADP + 2\ [P]i \longrightarrow 2\ \text{Piruvato} + 2\ NADH + 2\ H^+ + 2\ ATP + 2H_2O$$

O piruvato formado neste processo também pode ser convertido a lactato através da seguinte reação:



Piruvato **Lactato**

Neste caso, a enzima lactato desidrogenase (LDH) é responsável por catalisar a redução do piruvato a lactato, e de forma simultânea o NADH é oxidado a NAD^+ (Salway, 2009). A manutenção da relação NADH:NAD^+ é importante já que o aumento desta relação aumentaria a síntese de diacilglicerol (DAG), principal ativador fisiológico da proteína quinase C (PKC) responsável pela fosforilação de subunidades da enzima NADPH oxidase. A produção de espécies reativas de oxigênio pela oxidase, em pequenas quantidades, tem função na sinalização metabólica, mas em grandes quantidades pode gerar dano oxidativo. Pacientes diabéticos estariam mais susceptíveis a este processo devido à ativação de PKC por DAG, provocada pela hiperglicemia (Reis, 2008).

Em relação ao controle dos níveis de glicemia, o fígado desempenha um importante papel, juntamente com os músculos e o tecido adiposo. Isto é possível devido às ações combinadas de três hormônios, a insulina, o glucagon e a adrenalina. A insulina sinaliza a estes tecidos que os níveis de glicose sanguínea estão superiores aos necessários, isso resulta na captação do excesso de glicose sanguínea e sua conversão em compostos de armazenamento, como glicogênio e triacilgliceróis. De maneira oposta, o glucagon sinaliza quando a glicemia está baixa. Assim, os tecidos respondem através da produção da glicose a partir da degradação do glicogênio e pela oxidação de lipídeos. Já a adrenalina, quando liberada, prepara os músculos, os pulmões e o coração para uma maior atividade celular/energética (Nelson e Cox, 2002). A tabela 1 relaciona os efeitos do glucagon e da insulina na glicemia, na qual se observam os efeitos opostos destes hormônios. Pacientes

portadores de diabetes e que não conseguem manter a glicemia dentro dos valores de normalidade apresentam níveis elevados de glucagon, o que estimula a glicogenólise e a gliconeogênese, por outro lado, o glucagon exerce relativamente pouco efeito sobre a utilização periférica da glicose. Desta forma, em pacientes diabéticos com deficiência de insulina ou resistência à insulina e hiperglucagonemia, observa-se um aumento na produção hepática da glicose, redução na captação periférica e diminuição da conversão da glicose em glicogênio no fígado (Hardman e Limbird, 2006).

Tabela 1. Efeitos dos hormônios glucagon e insulina na glicemia.

Insulina	**Glucagon**
↑ Captação glicose (músculo, fígado)	↑ Degradação do glicogênio
↑ Síntese de glicogênio (fígado, músculo)	↓ Síntese do glicogênio (fígado)
↓ Degradação de glicogênio (fígado, músculo)	↓ Gliconeogênese (fígado)
↑ Glicólise, produção de acetil-CoA	↓ Glicólise (fígado)
↑ Síntese de ácidos graxos (fígado)	
↑ Síntese de triacilglicerol (tecido adiposo)	

1.3 Diabetes e Fitoterapia

A utilização de plantas medicinais para tratamento, cura ou prevenção de doenças, é uma das mais antigas formas da prática medicinal da humanidade (Veiga Junior, 2005). Isto é parcialmente atribuído à grande variedade de espécies vegetais existentes na flora mundial. Estima-se que cerca de 80% da população mundial utilize plantas como primeiro recurso terapêutico. Entre as diversas espécies vegetais de interesse medicinal, estão

as plantas do gênero *Bauhinia*, que pertencem à família Leguminosae. Este gênero de plantas é encontrado principalmente em áreas tropicais do planeta e compreende cerca de 300 espécies. No Brasil, as plantas do gênero *Bauhinia* são conhecidas popularmente como “pata-de-vaca”, e suas folhas, caules e raízes são utilizados como chá e em outras preparações para o tratamento de diversas enfermidades, entre estas o diabetes mellitus (da Silva e Cechinel Filho, 2002).

A *Bauhinia forficata* (BF) é a espécie de *Bauhinia* mais utilizada como antidiabético fitoterápico no Brasil (Volpato, 2008). Os primeiros relatos da sua possível atividade hipoglicemiante em pacientes diabéticos foram feitos por Juliani (1929; 1931). Estudos fitoquímicos e farmacológicos apontam como constituintes principais desta planta glicosídeos esteroídicos, triterpenos, lactonas e flavonoides (da Silva e Cechinel Filho, 2002). Embora existam algumas contradições a respeito dos efeitos de *B. forficata*, em geral os resultados relatam propriedades terapêuticas, atribuídas principalmente à presença de flavonoides. A análise fitoquímica realizada por Pizzolatti et al. (2003) confirmou a dos mesmos nas folhas de BF, destacando a kaempferitrina. Alguns estudos têm sido realizados na tentativa de atribuir os efeitos antidiabéticos dos extratos de folhas de BF a este composto (Pinheiro, 2006). Estudo realizado por Jorge et al (2004) atribuiu à kaempferitrina um efeito agudo na redução da glicose sanguínea em ratos diabéticos e estímulo na fixação da glicose no músculo de ratos normais, de forma tão eficiente quanto a insulina.

1.4 Utilização de *Bauhinia forficata* no diabetes experimental

Embora a utilização popular de BF seja bastante conhecida, estudos científicos com resultados consistentes sobre o real potencial hipoglicemiante desta espécie são escassos e muitas vezes contraditórios. Em um estudo conduzido por Pepato et al. (2002), foi avaliada a atividade antidiabética de uma decocção de BF (150 g folhas/L) em ratos diabéticos tratados por 31 dias. Os ratos diabéticos tratados com a decocção apresentaram uma significativa

redução da glicose sérica em comparação com animais diabéticos que não receberam o tratamento. O mesmo grupo de pesquisa, em 2004, conduziu um estudo com o objetivo de avaliar a toxicidade da BF em ratos diabéticos. Após 33 dias de administração de uma decocção de BF, foram avaliados os seguintes parâmetros de toxicidade sérica: lactato desidrogenase, creatina quinase, amilase, enzima conversora de angiotensina e bilirrubina. Através destes parâmetros os autores concluíram que a administração da decocção aquosa de *B. forficata* pode ser um tratamento potencial para diabetes e não produz efeitos tóxicos mensuráveis com os marcadores utilizados.

Lino et al. (2004) investigaram a atividade antidiabética dos extratos aquosos, etanólico e hexânico de BF, em um modelo de diabetes induzido por aloxano em ratos. Foram avaliados parâmetros como: glicose plasmática, triglicerídeos, colesterol total, HDL e LDL colesterol. Após um tratamento com duração de 7 dias nas doses de 200 ou 400 mg/Kg os animais apresentaram reduções significativas na glicemia, de triglicerídeos, de colesterol total e HDL em comparação com controles diabéticos. Em concordância, Silva et al. (2002) estudaram o efeito agudo da fração *n*-butanol de BF (400, 500, 600 e 800 mg/kg) sobre a glicemia de ratos. A administração oral das maiores doses desta fração conduziu a uma significativa redução da glicemia em ratos diabéticos, com o efeito máximo (cerca de 30%) observado na dose de 800 mg/kg após 1 hora de administração, sendo este perfil mantido durante as próximas 3 horas. Outro estudo realizado por 7 dias com a dose de 200 mg/Kg de extratos secos de BF também demonstrou potencial hipoglicemiante (Cunha, 2010).

Alguns autores têm atribuído o efeito hipoglicemiante dos extratos das folhas de BF ao seu flavonoide principal, o kaempferol-3,7-O-(R) – diramnosídeo, ou kaempferitrina (de Sousa et al., 2004), cuja estrutura está mostrada na figura 1.

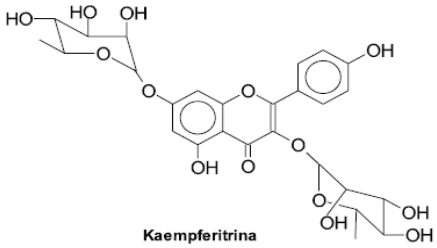


Figura 1. Estrutura química da kaempferitrina (da Silva e Cechinel Filho, 2002).

De Sousa et al (2004) realizaram um estudo agudo com o objetivo de investigar os efeitos da kaempferitrina na glicemia de ratos diabético-induzidos por aloxano. Para tanto, utilizaram a fração n-butanol de um extrato de BF. Foi observado um efeito hipoglicemiante em todas as doses testadas (50, 100 e 200 mg/kg). Em relação ao potencial antioxidante *in vitro*, o extrato mostrou alta reatividade com o radical 1,1-difenil-2-picrilhidrazil (DPPH), além de *in vivo* diminuir a atividade da mieloperoxidase e a peroxidação lipídica.

Volpato et al (2008) conduziram um estudo com o objetivo de investigar os efeitos do tratamento com o extrato aquoso de folhas de BF (500 a 100 mg/kg), no qual foi avaliado seu efeito sobre a glicemia, além de outros parâmetros. Contraditoriamente aos estudos anteriores, a administração de *Bauhinia forficata* não interferiu na glicemia de animais diabéticos. Seus resultados corroboram o anterior de Coimbra-Teixeira et al. (1992), que usaram extrato alcoólico de BF. Reforçando estes resultados, a dose de 400 mg/kg da fração *n*-butanol de BF, usada por Silva et al. (2002), também não produziu alteração da glicemia em ratos diabéticos Considerando os relatos contraditórios do potencial hipoglicemiante de BF, os poucos relatos sobre sua atividade antioxidante *in vivo* e toxicidade hepática, e a inexistência de estudos que avaliem os efeitos de BF sobre o metabolismo hepático da glicose, é que o presente trabalho foi delineado.

2. OBJETIVO

2.1. Objetivo Geral

Avaliar os efeitos metabólicos e antioxidantes de *Bauhinia forficata* (BF) em ratos diabéticos.

2.2. Objetivos específicos

O presente projeto objetivou avaliar de modo específico:

a) As vias metabólicas da glicose no fígado diante da administração *ex vivo* do extrato etanólico de BF;

b) A atividade hipoglicemiante *in vivo* aguda e subaguda do extrato etanólico de BF;

c) O potencial antioxidante *in vitro* e *in vivo* do extrato etanólico de BF;

d) O potencial hepatotóxico *in vivo* do extrato etanólico de BF;

e) Os efeitos microscópicos (histológicos) do extrato de BF sobre o fígado e o pâncreas de ratos.

3. ARTIGO CIENTÍFICO

ETHANOLIC EXTRACT OF Bauhinia forficata: EVALUATION OF THE METABOLIC AND ANTIOXIDANT EFFECTS IN DIABETIC RATS

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3.1 Introduction

Diabetes is a group of metabolic diseases characterized by hyperglycemia and associated complications, such as dysfunction and failure of the eyes, kidneys, nerves, brain, heart, and blood vessels. Diabetes may result from defects in secretion and/or insulin action involving specific pathogenic processes, including destruction of pancreatic beta cells, insulin resistance, and insulin secretion disorders (MHO (MS), 2006). The estimated prevalence of diabetes in the under-40 population is 11%, representing about 5.5 million people (MHO (MS) 2006). Diabetes is associated with high morbidity and mortality, with a significant loss in quality of life. It is a major cause of mortality related to renal failure, lower limb amputation, blindness, and cardiovascular disease.

The most common forms of diabetes are type 1 (T1DM), formerly known as juvenile diabetes, which comprises about 10% of cases, and type 2 (T2DM), previously known as adult-onset diabetes, which comprises about 90% of cases (MS, 2006). T1DM is treated with insulin, which should be instituted as soon as the disease is diagnosed; T2DM is treated with oral hypoglycemic agents and, in some cases, it becomes necessary to administer insulin, while exercise and diet are ancillary measures to control the disease. More than 400 plant species have been reported to possess hypoglycemic activity, but only a few have been investigated (Silva et al., 2000). Among the plants commonly used to treat diabetes is the genus *Bauhinia*, particularly *B. forficata* (BF), known as “cow’s foot”, which is used as a tea in folk medicine. The phytochemical analysis performed by Pizzolatti et al. (2003) confirmed the presence of flavonoids in the leaves of BF, highlighting kaempferitrin as the most active hypoglycemic compound.

Although studies have shown that 30-day administration of a decoction of BF leaves reduces plasma glucose in diabetic animals (Pepato et al., 2002), some researchers have turned to studies of extracts obtained from the leaves of BF, to concentrate active compounds such as kaempferitrin (Cunha et al. 2006; Lino et al. 2004, Silva et al. 2002). Silva et al. (2002) showed that the *n*-

butanolic extract of BF reduces acute blood glucose in alloxan-induced diabetic rats and George et al. (2004) suggest kaempferitrin functions as an insulin-mimic in diabetic rats. Other authors have not observed hypoglycemic activity in BF preparations (Coimbra-Teixeira et al., 1992; Volpato et al., 2008). The *in vitro* antioxidant effect of kaempferitrin (de Sousa et al., 2004) may be important in the diabetes model, as experimental and clinical studies suggest oxidative stress plays a role in the pathogenesis of both types of diabetes (Maritim et al., 2003). Tzeng et al. (2009) demonstrated that kaempferitrin promotes activation of the classical insulin pathway to activate GLUT4 translocation and stimulate continuous adiponectin secretion. Thus, it promotes peripheral sensitivity to insulin, but no studies have evaluated the effects of BF on hepatic metabolism of glucose. In this context, there are many aspects of BF that need to be clarified. In the absence of consensus regarding BF bioactivities, we aimed to study the metabolic, hypoglycemic, and antioxidant effects of ethanolic extract of BF leaves in diabetic rats.

3.2 Materials and Methods

All protocols were approved by the Ethics Committee for Animal Experimentation (CEUA) of the Biological Science Sector of UFPR (certificate number 577) and performed according to the guidelines regarding the ethical use of laboratory animals.

3.2.1 Botanical material

The botanic material, previously identified and deposited in the Botanical Museum of Curitiba (# 384637), was collected in the Curitiba Municipality Garden and dried under controlled temperature. To concentrate kaempferitrin, we performed an extraction by maceration, in which dried leaves of *B. forficata* were successively extracted with solvents in increasing order of polarity, namely hexane (Hex), dichloromethane (DCM), ethyl acetate (AcOEt), and ethanol (EtOH). The extracts were analyzed by nuclear magnetic resonance (NMR) H^1

to confirm the presence of kaempferitrin, which was concentrated in the ethanolic extract. The work of Pizzolatti et al. (2003) was used as the basis of the compounds identification.

3.2.2 *In vitro* free radical scavenging activity

The reactivity of isolated extracts of *B. forficata* with the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) was evaluated by measuring the absorbance changes at 517 nm as described by Chen et al. (1999), with some modifications. Seven concentrations, from 1 to 1000 µg mL^{-1}, were tested in the BF extracts obtained with the solvents: hexane, dichloromethane, ethyl acetate, and ethanol. The reaction system was composed of 750 µL of test solution and 250 µL of methanol DPPH solution (1.0 mg in 25 mL). After 30 s, the decrease in absorbance was measured. The reductant ascorbic acid (50 µg mL^{-1}) was used as a positive control, while distilled water was used as a negative control. This determination of *in vitro* antioxidant activity was used to identify the extract to be tested *in vivo*.

3.2.3 Induction of experimental diabetes

Adult male Wistar rats, weighing between 180 and 200 g, were kept in a temperature-controlled room (20–23°C) and fed *ad libitum* with a balanced commercial chow and water. Diabetes was induced by intraperitoneal administration of 50 mg kg^{-1} of streptozotocin (STZ) diluted in citrate buffer 10 mM (pH 4.5) after fasting for 12 h (Schreiber et al, 2012). Administration of this toxin produces a diabetic status compatible with type I diabetes; disease status was confirmed by measuring glycemia (via tail puncture) with reactive strips in a glucometer (Accu-Chek®, Roche). The animals were considered diabetic when the glycemia was >250 mg dL^{-1}. Experiments (section 3.2.4) or treatments (section 3.2.5) were initiated 7 days after STZ administration, a period necessary for disease stabilization.

3.2.4 Liver perfusion

The effects of *B. forficata* in hepatic glucose metabolism were evaluated in perfused livers in an *ex vivo* method. For the surgical procedure, rats were anesthetized by intraperitoneal injection of ketamine (100 mg kg^{-1}) and xylazine (10 mg kg^{-1}). Hemoglobin-free and non-recirculating perfusion was performed as described by Bracht et al. (2003). After cannulation of the portal and cava veins, the liver was positioned in a plexiglass chamber. Flow was maintained by a peristaltic pump (Minipuls 3, Gilson, France) between 28 and 35 mL min^{-1}, depending on liver weight. The perfusion fluid was Krebs/Henseleit-bicarbonate (KH) buffer (pH 7.4) containing 0.025% bovine serum albumin, saturated with a mixture of oxygen and carbon dioxide (95:5) by means of a membrane oxygenator with simultaneous temperature adjustment (37°C). The composition of the KH buffer was as follows: 115 mM NaCl, 25 mM $NaHCO_3$, 5.8 mM KCl, 1.2 mM Na_2SO_4, 1.18 mM $MgCl_2$, 1.2 mM NaH_2PO_4, and 2.5 mM $CaCl_2$. Substrates and extracts of BF were added to the perfusion fluid according to the following protocols:

(a) 16-h fasted rats (n = 3–4), whose livers were perfused with substrates L-glutamine (2.5 mM) and ammonium chloride (0.6 mM) from the 10^{th} to the 82^{nd} minute of perfusion, and ethanolic extract of BF (40 mg L^{-1}) concurrently from the 50^{th} minute.
(b) Fed rats (n = 3), whose livers were perfused with KH for 50 min, with BF infusion (40 mg L^{-1}) from the 10^{th} to the 40^{th} minute.

Samples of the effluent perfusion fluid were collected every 2 or 4 min and analyzed by standard enzymatic procedures for glucose, lactate, and pyruvate (Bergmeyer, 1974). The oxygen concentration in the outflowing perfusate was monitored continuously with a Teflon-shielded platinum electrode positioned in the plexiglass chamber at the exit of the perfusate (Bracht et al., 2003). Metabolic rates were calculated from input-output differences in total flow rate and were referred to the wet weight of the liver.

3.2.5 Rat groups, treatment schedule, and sample collection

To check the hypoglycemic and antioxidant effects of BF extract, an *in vivo* experiment was performed in normoglycemic (control) and diabetes rats. The treatment started 7 days after STZ administration and was conducted for the next 7 days, with 4 groups of animals (n = 6): (1) diabetic rats treated with extract, (2) diabetic rats treated with vehicle (tween 80 plus water), (3) normoglycemic rats treated with extract, and (4) normoglycemic animals treated with vehicle. For some experiments a positive control group was used, composed by (5) diabetic rats treated subcutaneously with 6.0 IU NPH insulin, once a day. Preliminary experiments with a dose of 150 mg kg^{-1} BF extract in diabetic rats for 7 days produced no hypoglycemic effect (data not shown); thus, we chose an orally administered dose of 300 mg kg^{-1} BF ethanolic extract. The variation in the rats' body weight was also measured during the treatment.

At the end of the treatment period, animals were anesthetized with intraperitoneal ketamine (100 mg kg^{-1}) and xylazine (10 mg kg^{-1}) and sample materials were collected (blood, liver, and pancreas). Blood was collected by cava vein puncture; the plasma was separated by centrifugation and kept at –20°C. After harvest, fragments of the liver and pancreas were immediately placed in 4% buffered formalin for histological studies, and part of the liver was stored at –80°C. The animals were euthanized by diaphragmatic puncture.

3.2.6 Evaluation of the hypoglycemiant potential of the *B. forficata* extract

Two experiments were performed each animal subsequently to evaluate the effects of BF extract on blood glucose: ***(a)*** Acute, in which blood glucose levels were measured before and 1 and 3 h after administration of the extract or vehicle; and ***(b)*** Subacute, after 7 days of treatment with extract or vehicle. In both experiments, glycemia was measured in tail vein blood with reactive strips and a glucometer.

3.2.7 Hepatic oxidative stress

To measure the activities of catalase (Cat), glutathione-S-transferase (GST), and superoxide dismutase (SOD), indicators of hepatocyte redox state, liver samples were homogenized and centrifuged at 10000 g for 20 min at 4°C. Enzyme activity in the supernatant was measured by established methods: Cat according to Aebi (1984), SOD by pyrogallol oxidation (Gao et al., 1998), and GST according to Habig et al. (1974). The rate of lipid peroxidation (LPO) was measured by the FOX method as described by Jiang et al. (1991). This technique quantifies the formation of hydroperoxides during lipid peroxidation. The concentration of reduced glutathione (GSH) was measured according to Sedlak and Lindsay (1968) with modifications. The results were expressed in activity by the amount of protein in homogenates, determined by the Bradford method (1976).

3.2.8 Plasma biochemistry

Hepatocyte integrity and blood protein glycosylation were assessed through plasmatic parameters. Measurements of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and fructosamine were performed using commercial kits (Kovalente, São Gonçalo – RJ - Brazil) in an automated system (Mindray BS-200).

3.2.9 Histology of the pancreas and liver

To complement the experiments described in sections 3.2.7 and 3.2.8 and to observe any changes induced by diabetes or treatment with BF extract, samples of the pancreas and liver were collected for histology. After fixation, samples were dehydrated in a graded series of ethanol before paraffin embedding. Thin sections (4 µm) were processed for histology and stained with hematoxylin and eosin (HE) (Culling et al., 1985). Groups were compared and

lesions were scored (0–3) as follows: 0, no change; 1, mild changes; 2, moderate changes; and 3, pronounced changes.

3.2.10 Determination of hepatic glycogen content

The determination of liver glycogen was performed as described by Kepler & Decker (1974), starting from 2.0 g of frozen tissue. The liver was minced with liquid nitrogen in a pestle and a 5× volume of 0.6 N perchloric acid was added to macerate the sample. The contents were transferred to a tube and homogenized in a Potter-Elvehjem homogenizer. The glucose content of the homogenate was measured and designated “basal glucose”. The hydrolysis of glycogen in this homogenate was achieved with the addition of 1.0 M potassium bicarbonate, 0.2 M amyloglucosidase, and acetate buffer, pH 4.8. The pH of the mixture was adjusted to 6.0. The tube was covered and placed in a 40°C water bath and stirred for 2 h. The reaction was stopped by addition of 0.6 N perchloric acid and the samples were centrifuged at 6000 rpm for 5 min at 4°C. Total glucose in the supernatant (final glucose) was determined with a commercial kit and a spectrophotometer at 505 nm. The difference between basal and final glucose was considered the glycogen content and expressed as glycosyl units.

3.2.11 Statistical analysis

Data are presented as mean ± standard error of the mean. The comparison between experimental groups was made by analysis of variance (ANOVA) with Bonferroni correction. Metabolites obtained in liver perfusion were assessed by nonparametric Student’s t test. Differences were considered significant when $p < 0.05$. The analyses were performed using the statistical program GraphPad Prism version 5.

3.3 Results

3.3.1 Analysis of the extracts obtained from the ethanolic extract of *B. forficata*

We successfully isolated the flavonoid in a butanol extract, although the flavonoid concentration was low, the butanol evaporated slowly, and it often emulsified (data not shown). Thus, an alternative to fractionation was sought, with different solvents. After extraction, the fractions were subjected to nuclear NMR ^{1}H to assess whether the fraction contained peaks characteristic of kaempferitrin, the main flavonoid of BF. The spectra of the DCM, AcOEt, and EtOH fractions are shown in Figure 1A and 1B. Comparison of the spectra confirmed that the extract of interest for our study was the ethanolic, in which the desired flavonoids, as kaempferitrin, were concentrated. Extraction of the ethanol fraction presented a yield about 2.5%.

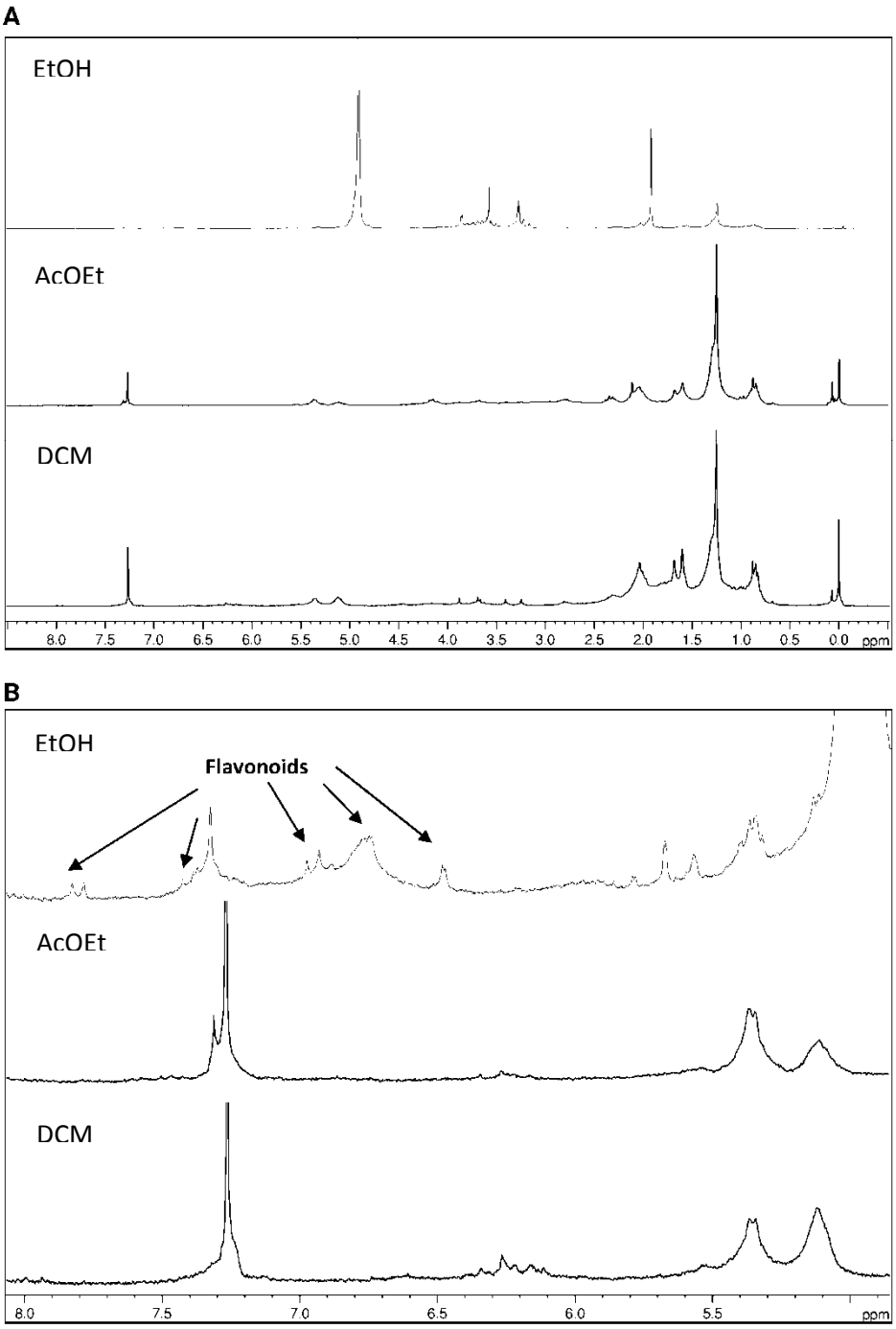

Figure 1. NMR ^{1}H of the *B. forficata* extracts in DCM (inferior), AcOEt (medium) and EtOH (superior) solvent. **(A)** The DCM and AcOEt extracts are similar, showing signals of aliphatic hydrogen, while the EtOH extract showed sugar signals in the region between 3.0 and 5.5 ppm. **(B)** Presence of flavonoids is shown in the EtOH spectra.

3.3.2 DPPH sequestration by *B. forficata* extracts

To determine the antioxidant capacity of the BF preparations, an *in vitro* test was performing with DPPH as the free radical. All preparations exhibited satisfactory sequestration of DPPH between 30 and 1000 µg mL^{-1}. However, the greatest antioxidant activity was found in the ethanolic extract, which showed even higher activity than the positive control (ascorbic acid) at concentrations above 30 µg mL^{-1}. These results are shown in Figure 2.

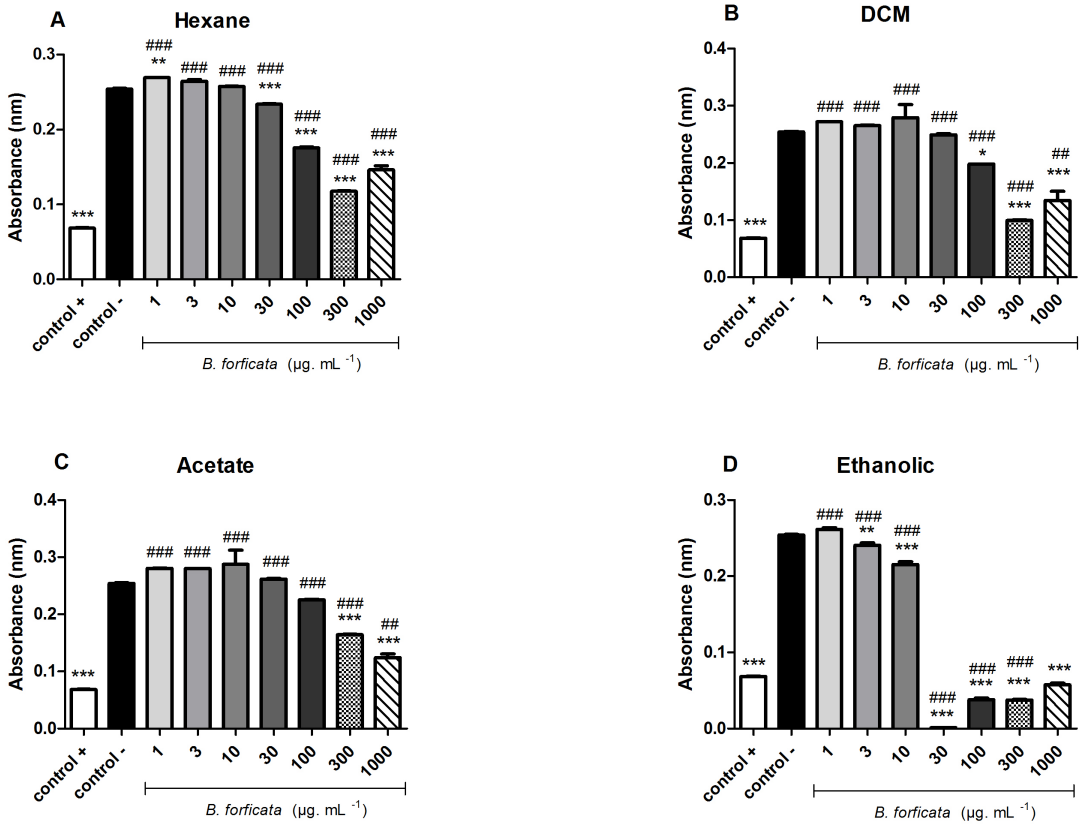

Figure 2. Activity of different extracts of *B. forficata* to neutralize the free radical DPPH: **(A)** hexane extract, **(B)** dichloromethane extract (DCM), **(C)** ethyl acetate extract and **(D)** ethanol extract, at different concentrations, compared to the negative (water) and positive control (ascorbic acid, AA - 50 µg.mL^{-1}). Symbols: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared to negative control (water). ### $p < 0.001$, compared to positive control.

3.3.3 Effects of *B. forficata* on liver glycogen catabolism and glycolysis

The first experiments tested the effects of BF on liver glycogen catabolism and glycolysis. Liver from fed rats perfused with substrate-free medium survived at the expense of glycogen degradation via glycogenolysis and oxidation of endogenous fatty acids (Scholz and Bücher, 1965). Under these conditions, the livers release glucose, lactate, and pyruvate because of glycogen catabolism. Figure 3 illustrates the responses of perfused livers to BF ethanolic extract infusion and the experimental protocol: after a pre-perfusion period of 10 min, the ethanolic extract of *B. forficata* (40 mg L^{-1}) was infused for 30 min, followed by an additional 10 min of extract-free medium perfusion. Four parameters were measured: glucose release, lactate and pyruvate production, and oxygen consumption. The livers of diabetic rats exhibited lower lactate and pyruvate production and increased oxygen consumption in comparison to normoglycemic rat livers (Figure 3). Pyruvate production was not changed by BF, but increased in controls when the extract infusion was ceased. Lactate production was only minimally reduced by BF infusion in control animals, but it was enough to cause a reduction in the lactate/pyruvate ratio (Table 1), a parameter that indicates the cytosolic NADH/NAD^+ ratio. Glucose release was slightly reduced by BF in normoglycemic rats but exhibited a greater reduction in the diabetic rats in the presence and absence of extract. Oxygen consumption and metabolite production with BF infusion are shown in Table 1. As noted, the BF ethanolic extract significantly reduced glucose release by about 164% in diabetic rats and the lactate/pyruvate ratio by about 60% in diabetic rats.

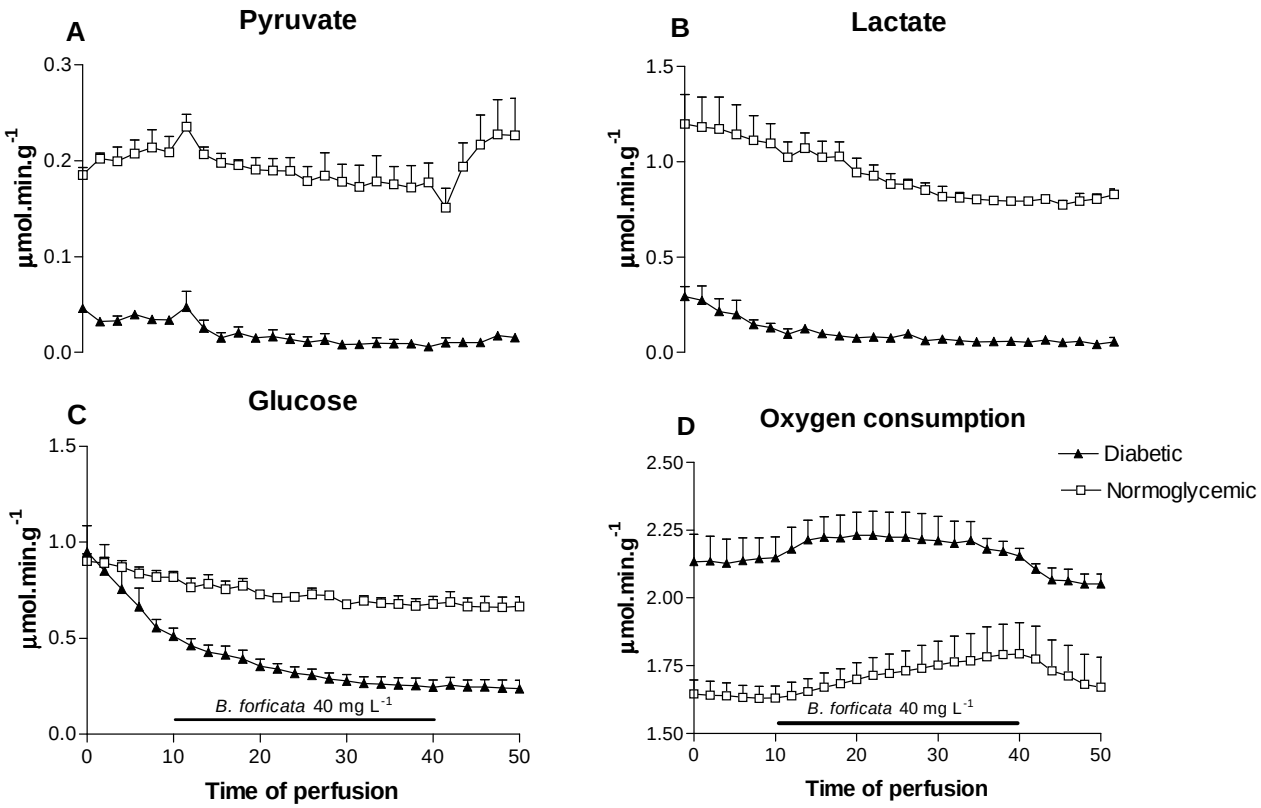

Figure 3. Hepatic production of metabolites (µmol g min^{-1}) in fed diabetic and normoglycemic rats (n = 3) during 50 minutes of monovascular liver perfusion. The BF extract was added to the perfusion liquid from the 10^{th} to the 40^{th} minute. **(A)** Production of pyruvate, **(B)** lactate, **(C)** glucose, and oxygen consumption.

Table 1. Influence of the *B. forficata* extract in the hepatic metabolism of fed normoglycemic and diabetic rats.

Parameter (µmol $min\ g^{-1}$)	**Animal groups**	
	Normoglycemic (n = 3)	**Diabetic (n = 3)**
Glucose production	-0.172 ± 0.045	-0.455 ± 0.006 *
Lactate production	-0.352 ± 0.131	-0.150 ± 0.043
Production of pyruvate	-0.027 ± 0.016	-0.028 ± 0.005
Oxygen consumption	0.142 ± 0.062	0.047 ± 0.036
Ratio lactate/pyruvate	13.0	5.3

The data represent the mean ± standard errors of the mean (n = 3) of the changes caused by the extract of *B. forficata*. The results were calculated as [final values at the end of the infusion period with BF] - [basal rates before infusion with BF]. Symbol: * $p < 0.05$ compared with the control by t-Student test.

3.3.4 Effects of *B. forficata* on liver gluconeogenesis

The experiments shown in Figure 4 tested the effect of BF ethanolic extract on liver gluconeogenesis from L-glutamine. In order to minimize interference by glycogen catabolism, livers from 16-h fasted rats were used. After a pre-perfusion period of 10 min in the absence of substrate, 2.5 mM L-glutamine plus 0.6 mM ammonium chloride were infused for 82 min and BF was added to the infusion with substrates from the 50th min. Ammonium chloride was added to accelerate L-glutamine catabolism because it stimulates glutaminase activity (Comar et al., 2010). Diabetic animals, even in the fasted state, showed a higher basal production of glucose and lactate when compared to the normoglycemic animals. L-Glutamine infusion caused an increase in glucose and lactate production in both normoglycemic and diabetic animals, but did not change pyruvate production. Glucose production was only minimally increased in diabetic animals in comparison to the basal values. In contrast, the increase in lactate production was more substantial in the diabetic animals. These scenarios changed after BF infusion (50th min). Pyruvate production experienced a higher and transitory increment at the start of extract infusion in normoglycemic and diabetic animals, but rapidly returned to basal values for both conditions. Lactate production was not changed by BF in the controls, but the extract reduced the increment of lactate production induced by L-glutamine in diabetic animals. Both the increment and reduction in metabolites due to BF infusion are shown in Table 2. The BF ethanolic extract significantly reduced lactate production by about 60% in diabetic animals while the lactate/pyruvate ratio was reduced by 76% and 131%, respectively, for normoglycemic and diabetic animals. However, the infusion of BF did not significantly change glucose production.

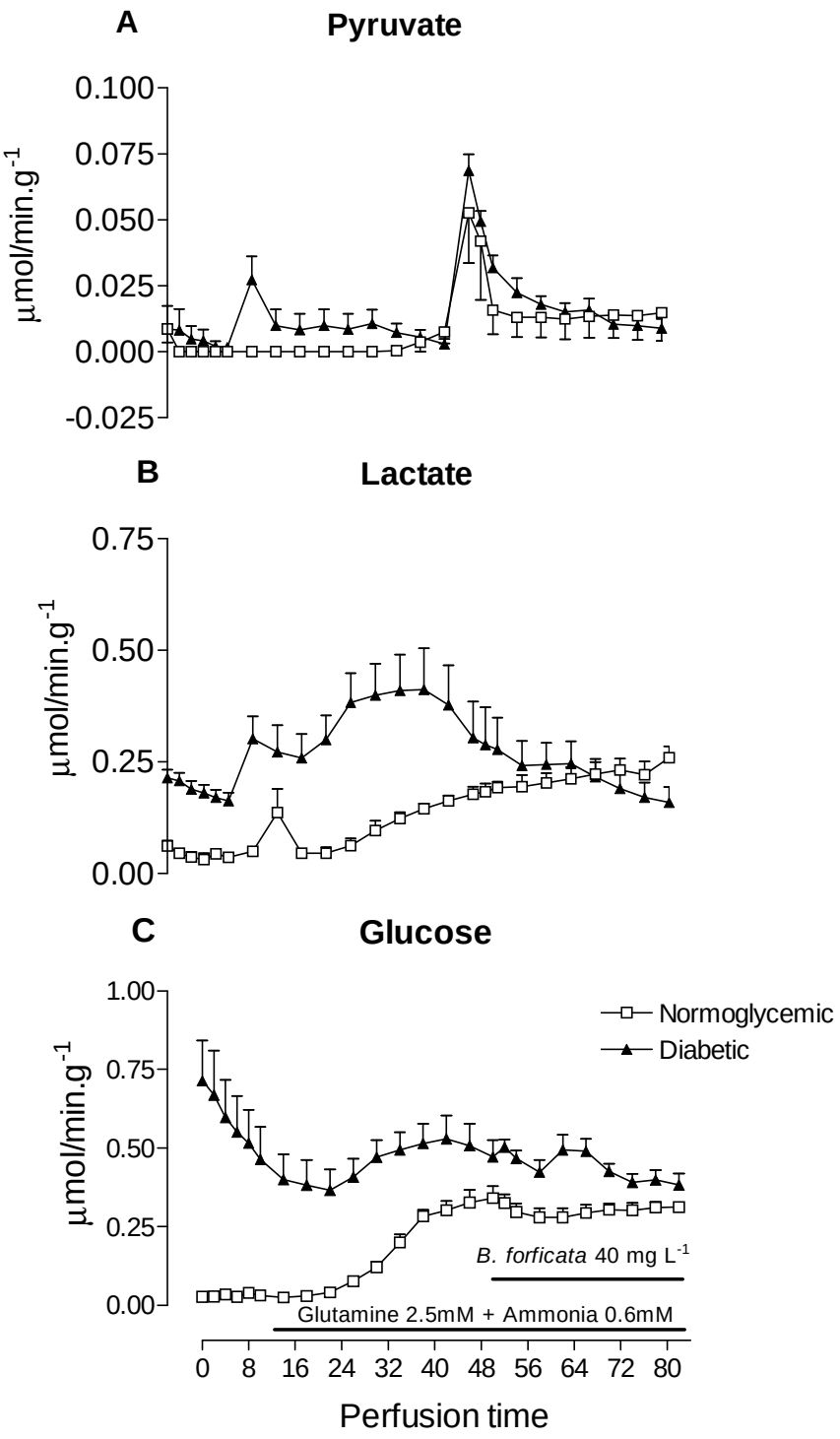

Figure 4. Hepatic production of metabolites (µmol g min^{-1}) in fasting diabetic (n = 4) and normoglycemic (n = 3) livers stimulated with the combination of L-glutamine 2.5 mM and ammonium chloride 0.6 mM. The infusion of BF extract occurred concomitantly from the 10^{th} to the 82^{nd} minute. **(A)** Production of pyruvate, **(B)** lactate and **(C)** glucose.

Table 2. Influence of the *B. forficata* extract in the L-glutamine metabolism in the liver of fasting normoglycemic and diabetic rats.

Parameter	(A) L-glutamine influence		(B) *B. forficata* influence	
(μmol min g^{-1})	Normoglycemic (n = 3)	Diabetic (n = 4)	Normoglycemic (n = 3)	Diabetic (n = 4)
Glucose production	0.236 ± 0.014	-0.084±0.097*	0.036 ± 0.004	-0.072 ± 0.047
Lactate production	0.089 ± 0.013	0.193 ± 0.074	0.091 ± 0.031	-0.163 ± 0.040**
Pyruvate production	0.003 ± 0.002	0.009 ± 0.002	0.013 ± 0.007	0.0239 ± 0.003
Ratio lactate/pyruvate	29.6	21.4	7.0	-6.8

The data represent the mean ± mean standard errors of the changes caused by L-glutamine infusion (column A) and the extract of *B. forficate* (column B). The first was calculated as [final values at the end of the L-glutamine infusion period] – [basal rates before L-glutamine infusion]; and the latter was calculated as [final values at the *B. forficata* infusion period] - [final values at the end of the L-glutamine infusion period]. Symbols: * $p < 0.05$ and ** $p < 0.05$ compared with the control under the same condition.

3.3.5 Evaluation of the hypoglycemic potential of the *B. forficata* extract

Considering the changes observed in the hepatic metabolic pathways of glucose, we determined the effects of BF extract (oral) in normoglycemic and diabetic rats. Animals were considered diabetic when blood glucose levels three days after STZ injection reached levels of >250 mg dL^{-1}. The mean blood glucose of diabetic animals at this time was 496 ± 30.3 mg dL^{-1}. The assessment of BF hypoglycemic potential was conducted acutely and subacutely in the same animals. In both situations, despite a reduction in glycemia in all animals, there was no significant reduction in the blood glucose of diabetic animals after 1 and 3 h, or after 7 days of administration of 300 mg kg^{-1} BF extract, when compared with normoglycemic rats. However, as expected, a significant reduction in the glycemia was observed in insulin-treated rats by ~25%, ~90%, and ~55% at 1 h, 3 h, and 7 days after treatment (Figure 5A-C).

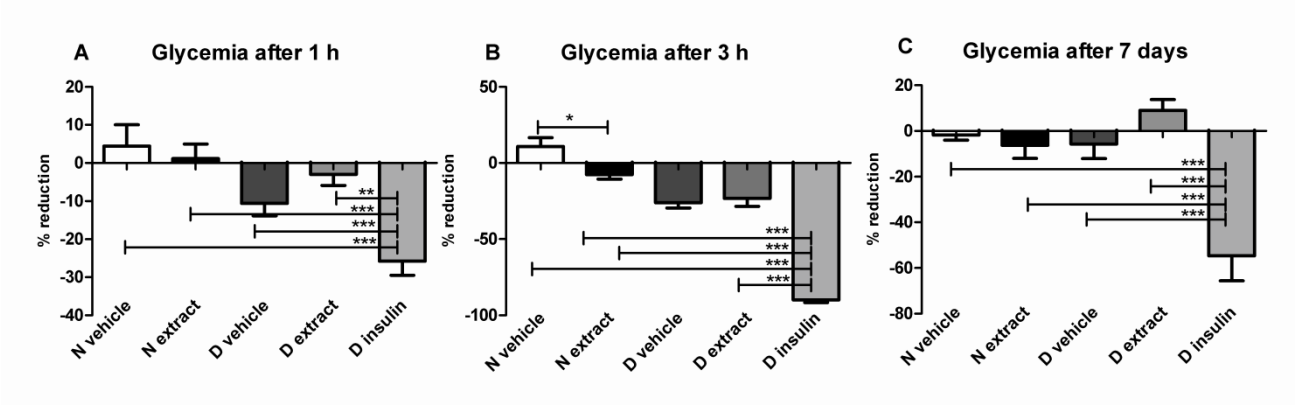

Figure 5. Changes in blood glucose of rats 1 h **(A)**, 3 h **(B)** and 7 days **(C)** after the treatment with 300 mg Kg the ethanolic extract of *B. forficata*, vehicle or insulin. The data are expressed as the percentage considering the pre-treatment blood glucose as 100% for each group. Legends: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, N = normoglycemic, D = diabetic.

3.3.6 Changes in body weight

To assess whether the BF influences body weight, animals were weighted at the beginning and after 7 days of treatment. The body weight gain of the normoglycemic group (17.3 ± 2.1g) was higher than in diabetic rats (12.9 ± 2.4 g), and the administration of BF extract did not improve this parameter in diabetic (8.2 ± 1.1 g) animals.

3.3.7 Oxidative stress in the liver

Considering the importance of oxidative stress in the pathogenesis of diabetes and the fact that hepatic biotransformation of xenobiotics such as BF extract can generate an oxidant situation, we evaluated the hepatic activity of superoxide dismutase (SOD), glutathione-S-transferase (GST), and catalase (Cat), the levels of reduced glutathione (GSH), and the rate of lipid peroxidation (LPO). Diabetic animals showed a decrease in SOD activity compared to normal animals, while BF administration restored enzyme activity in diabetic animals (Figure 6A). However, the same was not observed for GST, which

exhibited reduced activity in diabetic animals treated with vehicle or extract when compared to normoglycemic rats (Figure 6B). The activity of Cat (Figure 6C) and the level of GSH (Figure 6D) were increased by the extract, but only in normal animals. LPO rates did not significantly differ between groups (Figure 6E). BF extract administration did not alter these hepatic parameters in diabetic rats.

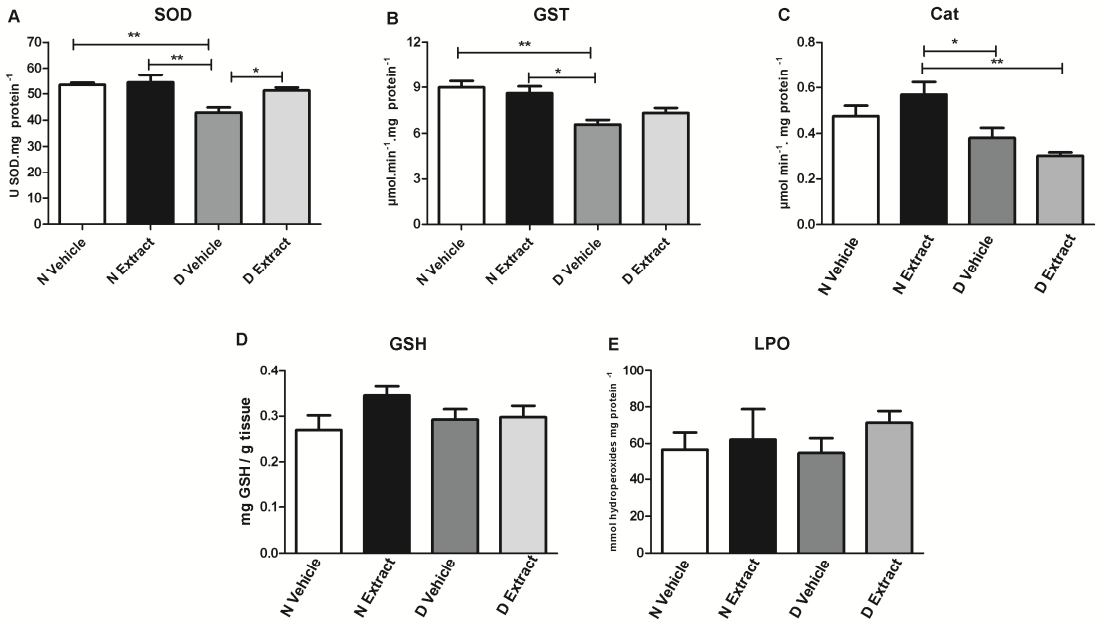

Figure 6. Oxidative stress parameters analyzed in liver of rats treated for 7 days with ethanolic extract of *B. forficata*: **(A)** activity of superoxide dismutase, **(B)** glutathione-S-transferase, **(C)** catalase, **(D)** reduced glutathione levels, and **(E)** rate of lipid peroxidation. Symbols: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, N = normoglycemic, D = diabetic.

3.3.8 Histology of the pancreas and liver

Histology of the pancreas was performed to evaluate changes induced by experimental diabetes. This analysis revealed that the streptozotocin model reduced by nearly half the number of Langerhans' islets in comparison to non-

diabetic animals (Table 3 and Figure 7). Counting of pancreatic islets was carried out in 4 fields at 100× magnification under a light microscope. The parameters evaluated and the scores assigned to each group are described in Table 3. There was no difference between diabetic animals treated with extract and diabetic animals treated with vehicle. Liver histology revealed no significant changes between experimental groups, indicating that neither diabetes nor the BF extract were able to produce alterations in the microscopic features of this organ.

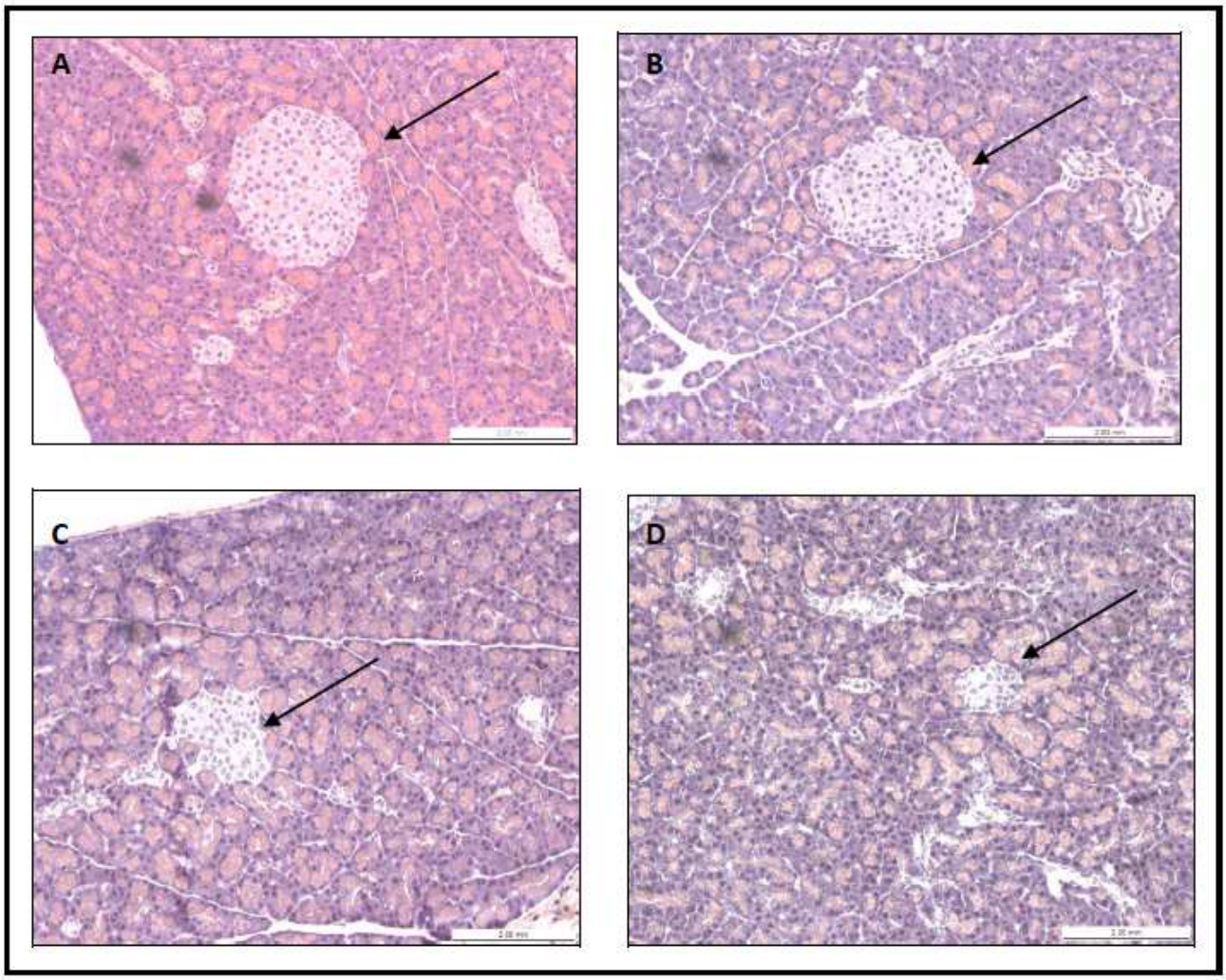

Figure 7. Pancreatic histology of rats treated for 7 days with the ethanolic extract of *B. forficata* or vehicle. The arrows indicate the islets of Langerhans in **(A)** normoglycemic rats treated with vehicle, **(B)** normoglycemic rats treated with extract, **(C)** diabetic rats treated with vehicle and **(D)** diabetic rats treated with the extract.

Table 3. Histological parameters evaluated in the pancreas of normoglycemic or diabetic rats treated for 7 days with vehicle or *B. forficata* extract (300 mg kg^{-1}).

Parameter	Animal Groups			
	N Vehicle	N Extract	D Vehicle	D Extract
Presence of vacuoles	0	1	2	2
Apoptosis	0	0	0	1
Presence of MMN	0	0	1	1
Presence of nucleolus	0	0	2	1
Organization islets	0	0	2	1
N^{Θ} Langerhans islets	3.7 ± 0.48	3.3 ± 0.58	1.8 ± 0.20***	1.8 ± 0.81***

Legend: 0, no changes; 1, slight alterations; 2, moderate changes; MMN, Monomorphonuclear; N = normoglycemic; D = diabetic. Symbol: *** $p < 0.001$ compared with the N vehicle. Counted islets were carried out in 4 fields at 100x magnification at optical microscopy.

3.3.9 Biochemical parameters

Both plasmatic transaminases showed abnormalities in BF-treated diabetic animals; alanine- (ALT) and aspartate aminotransferase (AST) levels increased 76% and 95%, respectively, in comparison to vehicle-treated normoglycemic animals. Interestingly, the vehicle-treated diabetic rats exhibited no alteration in these enzymes. Since no hepatic alteration was visible by light microscopy (section 3.3.8), we speculate the increase in transaminases may be related to a specific cellular or molecular alteration caused by the interaction between the diabetic condition and BF treatment. Plasma fructosamine did not differ between groups (Table 4).

As glucose release was reduced in the perfused liver of fed diabetic rats, an *in vivo* experiment was performed to assess liver glycogen in these and normoglycemic animals. The results are shown in the Table 4. The liver of diabetic rats presented very low glycogen content: less than 20% of the content in the normoglycemic rats. The treatment of animals with *B. forficata* extract

increased liver glycogen content by about 100% in the diabetic condition, but was still less than the controls.

Table 4. Biochemical parameters (mean ± SEM) analyzed in normoglycemic or diabetic rats (n = 6) treated for 7 days with ethanolic extract *of B. forficata* (300 mg kg^{-1}) or vehicle.

Parameter	Animal Groups			
	N Vehicle	**N Extract**	**D Vehicle**	**D Extract**
Plasma ALT (U L^{-1})	48.5 ± 8.42	41.8 ± 8.37	53.5 ± 7.92	85.4 ± 22.11**
Plasma AST (U L^{-1})	66.83 ± 10.91	64.6 ± 11.09	68.4 ± 12.11	130.7 ± 32.57***
Plasma Fructosamine (µmol L^{-1})	343 ± 68.51	324.5 ± 51.85	307 ± 35.06	291.8 ± 63.37
Liver Glycogen (µmol of glycosyl per mg of liver weight)	60.23 ± 13.54	93.83 ± 11.09	11.39 ± 3.97* ###	21.88 ± 3.81###

Legend: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to N vehicle; ### $p < 0.001$ compared to N extract; N = normoglycemic, D = diabetic.

3.4 Discussion

The plants of the genus *Bauhinia sp.*, especially *B. forficata*, are popularly identified as natural hypoglycemic agents, indicated for the treatment of diabetes; however, scientific data regarding this and other activities are scarce in the literature. Some authors have investigated the hypoglycemic effect of *B. forficata* (Pepato et al., 2002; da Cunha et al., 2010), but the hepatic glucose pathways were not studied. This is the first report of the effects of BF extract in the hepatic metabolism of glucose. We evaluated the effects of BF on glycogenolysis and glycolysis in perfused fed rats; while perfused liver of fasted rats were stimulated with L-glutamine to investigate the effects of BF on gluconeogenesis.

Significant metabolic differences were observed between diabetic and normoglycemic rats in fasted and fed states. The liver of perfused diabetic rats exhibited higher glucose production under fasting conditions (Figure 4C), but similar production of glucose under fed conditions (Figure 3C) when compared to normoglycemic rats. Glycolysis was also reduced in the diabetic condition, as evidenced by the decrease in lactate and pyruvate release. Then increased oxygen consumption in diabetic rats could be the result of compensatory energy production from the aerobic consumption of fatty acids. Glucose levels in fed rats are compatible with the reduced glycogen level found in the liver of diabetic rats (Table 4). In the absence of insulin, glycogen synthesis ceases and glucogenolysis begins; thus, more glucose is exported from the liver to the blood, increasing hyperglycemia even more (Salway, 2009). Low glycogen accumulation has also been described in STZ-induced diabetic rats (da Cunha et al., 2010; Haritha et al., 2013; Marzouk et al., 2013; Bavarva and Narasimhacharya, 2013). Liver perfusion reduced glucose production more quickly in diabetic rats than in normoglycemic rats, probably because the control livers released glucose from accumulated glycogen (glycogenolysis), while diabetic rats release glucose via gluconeogenesis through endogenous substrate.

The infusion of BF in the perfused liver of diabetic rats reduced glucose release by 63%, but it did not affect glycolysis. Thus, the reduction in the glucose release seems to be a consequence of the depletion of glycogen stores, which were very low in these animals. The higher production of hepatic pyruvate in diabetic animals, possibly associated with increased catabolism of aminoacids in this condition (Salway, 2009), was not modified by BF infusion.

The effects of BF extract infusion on glucose production in fasted rat livers were less extensive than in fed condition. Since the basal glucose release (Figure 4C) in fasted diabetic rats (0.71 ± 0.12 μmol min g^{-1}) was significantly higher than in normoglycemic rats (0.027 ± 0.01 μmol min g^{-1}) and the glycogen content was very low, the released glucose is probably the product of increased gluconeogenesis. Higher gluconeogenesis in the absence of substrate infusion is a phenomenon that implicates an endogenous source of substrates, mainly gluconeogenic amino acids, particularly alanine and glutamine (Salway, 2009). Thus, the perfused liver releases a considerable concentration of amino acids

from proteolysis, which is enough to sustain gluconeogenesis in the diabetic rat liver (Zwiebel, 1990). For this reason, the increment in glucose production triggered by L-glutamine in diabetic rats was significantly lower than in normoglycemic rats, since its basal level was already elevated. However, concomitant infusion of BF with L-glutamine did not change glucose production in normoglycemic and diabetic fasted rats. Thus, the suggestion of Pepato et al. (2002) that BF could act in gluconeogenesis as the biguanines do was not confirmed by our data, at least when L-glutamine was the gluconeogenic source.

The livers of rats with type 1 diabetes respond differently to glutamine, and the differences are characterized mainly by an acceleration of metabolic flux during the initial stages of the amino acid infusion (de Oliveira, 2006). Consistent with de Oliveira (2006), the increment in lactate production triggered by L-glutamine was higher in diabetic rats (Figure 4B). BF infusion inhibited lactate production and stimulated pyruvate production; thus, the extract altered the cytosolic redox state since this indicated an accentuated reduction in the $NADH/NAD^+$ ratio. Despite the controversy surrounding the prevalence of lactic acidosis in patients with diabetic ketoacidosis (DKA), a correlation has been proposed between lactatemia and lactic acid production in this condition (Fulop et al., 1976; Cox et al., 2012). Cox et al. (2012) noted that patients with DKA presented seric lactate around 3.5 mmol L^{-1}. Thus, our data could suggest that the higher lactatemia in diabetes is, at least in part, originating from the hepatic metabolism of aminoacids such as L-glutamine.

Despite the reduction in glucose production induced by BF infusion in the livers of fed diabetic rats (Table 1), glycemia did not change, either acutely or subacutely. The scientific evidence for the BF effects on glycemia is controversial. Our results corroborate those of Coimbra-Teixeira et al. (1992) who used BF alcoholic extract and those of Volpato et al. (2008) who used an aqueous extract (500–1000 mg kg^{-1}); however, our results differ from those reported by Menezes et al. (2007) and Silva et al. (2002). The last group of authors observed acute hypoglycemia after treatment with 500 and 600 mg kg^{-1} of BF *n*-butanol extract. These doses, however, are higher than that used in this work (300 mg kg^{-1}). Reinforcing our results, the dose of 400 mg kg^{-1} in their

study also failed to reduce the glycemia in diabetic rats (Silva et al., 2002). To correlate the effects of glucose metabolism observed within the hepatic direct infusion of BF extract (perfusion technique) and the glycemia observed after oral administration is difficult, because we have no data regarding BF absorption rates. Gastrointestinal absorption of the main active compound of BF, kaempferitrin, has not been reported. It is possible that the amount of absorbed compound from the dose used in this study (300 mg kg^{-1}) is lower than the concentration infused into the liver (40 mg L^{-1}). The present data is insufficient to verify this hypothesis. However, considering the high glycemia observed in the diabetic rats (496 $\pm$ 30.3 mg dL^{-1}), we can speculate that the kaempferitrin in the BF extract was insufficient to reduce such high glycemic levels. This hypothesis is based on the observations of George et al. (2004), who estimated that kaempferitrin is 106-fold less potent than insulin. The reduction of insulin availability in diabetic rats was indirectly indicated by pancreas histology. The number and organization of pancreatic islets in diabetic animals were clearly decreased, with a reduction of about 50% in the number of Langerhans islets in comparison to normoglycemic animals. Non-significant improvement was observed in diabetic rats treated with the BF extract.

In this study, we opted to work with the ethanolic extract of BF leaves, in which NMR H^1 confirmed the presence of kaempferitrin. The DPPH *in vitro* assay showed that the chosen extract demonstrated antioxidant potential, a characteristic attributed to the presence of this flavonoid. Despite the strong *in vitro* antioxidant activity of the BF ethanolic extract, oral treatment with BF only slightly improved hepatic antioxidant parameters in diabetic rats, consistent with previous data (Khalil et al., 2008). SOD activity, reduced in diabetic animals, was the only antioxidant activity restored by BF extract. The extract also increased Cat activity and GSH levels in normoglycemic rats, but not in diabetic animals. The reduction of hepatic GST in the diabetic rats was consistent with the reduction of this enzyme activity in HepG2 cells (Raza and John, 2012) and in rat liver, both treated with STZ (Marzouk et al., 2013). However, BF treatment did not improve GST activity in diabetic rats.

Seven days BF treatment of diabetic rats was not able to assist these animals to gain weight as the control group. Body weight must be evaluated in patients with uncontrolled diabetes, as well as the plasmatic transaminases;

these parameters are markers of the progression of uncontrolled diabetes to metabolic syndrome (Saligram et al., 2012; Xourafas et al., 2012). Our data showed that the BF extract and STZ-induced diabetes in isolation did not alter transaminase activity, while the combination of these factors significantly increased ALT and AST. These results may indicate a hepatotoxic effect. Since this toxicity was not reflected in the liver histology, it is possible that hepatocyte membrane permeability was affected without changing membrane integrity, allowing extravasation of transaminases to the blood. In contrast, plasmatic fructosamine did not change with BF treatment. This result was expected since fructosamine forms glycated serum proteins (Armbruster, 1987), thus inferring changes in glycemia over a period of two to three weeks, but the present experiment was conducted for only one week.

In conclusion, ethanolic extract of BF produced interesting metabolic effects in the liver, with differences between the fed and fasted states. The effects on glucose metabolism were more extensive in the fed state, while lactate interference was more evident with L-glutamine metabolism in the fasted state. BF treatment caused a minimal increase in the liver glycogen content of diabetic rats, but not enough to restore the animals to control levels, and with no change in glycemia. Since this is the first study to demonstrate the effects of BF on hepatic metabolism, additional, longer-term studies are necessary to better assess its metabolic and toxicological effects, including analysis of sources of gluconeogenesis other than L-glutamine.

3.5 Acknowledgments

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4. CONSIDERAÇÕES FINAIS

Diante dos resultados obtidos neste trabalho, podemos concluir que:

● O extrato etanólico de BF produziu efeitos metabólicos no fígado de animais alimentados e em jejum, sendo que os efeitos sobre o metabolismo da glicose foram mais extensos em animais alimentados, enquanto que a interferência na produção de lactato foi mais evidente em fígado de animais em jejum estimulados com L-glutamina.

● O extrato etanólico de BF na dose e período utilizados, não foi capaz de reduzir a glicemia de animais diabéticos tanto de forma aguda como subaguda.

● Embora o extrato etanólico de BF tenha apresentado potencial antioxidante *in vitro,* o mesmo efeito foi observado de maneira mais atenuada *in vivo*.

● A administração do extrato etanólico de BF provocou um aumento nas transaminases hepáticas em animais diabéticos, podendo indicar um possível efeito tóxico pela combinação do extrato com o modelo de diabetes utilizado.

● A administração do extrato etanólico de BF não provocou alterações histológicas no fígado e pâncreas de animais diabéticos e normoglicêmicos tratados com o mesmo.

● Este é o primeiro estudo que demonstra os efeitos da BF no metabolismo hepático. Assim, estudos adicionais, de longo prazo, são necessários para melhor avaliar os seus efeitos metabólicos e toxicológicos.

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